

Please delete the paragraph on page 1 under "Cross Reference to Related Applications" and insert:

B2 This application claims priority to U.S. Serial No. 08/838,384 (abandoned), filed April 8, 1997, U.S. Serial No. 60/015,066, filed April 9, 1996 and to U.S. Serial No. 60/118,764, filed February 5, 1999.

At page 1, lines 18 - 25 and page 2, lines 1 - 8, please delete the paragraph and insert:

B3 During its early development, the murine allantois consists of an inner core of mesoderm and an outer layer of squamous epithelium referred to as a mesothelium. The allantois undergoes two major developmental processes: (i) maturation and fusion with the chorion to become the umbilical component of the chorioallantoic placenta, and (ii) vascularization, forming an artery and a vein that permit within the chorionic disk the exchange of nutrients, metabolic wastes and gases with the mother during fetal gestation (K.M. Downs and R.L. Gardner, Development 121:407-416, 1995; K.M. Downs and C. Harmann, Development 124:2769-2780, 1997; K.M. Downs, et al., The Murine Allantois. In Current Topics in Developmental Biology (eds. R. Pedersen and G. Schatten). New York: Academic Press. 39:1-33, 1998; K.M. Downs, supra, 1998).

At page 15, lines 3 - 27 and page 16, lines 1 - 2,
please delete the paragraph and insert:

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The mouse is an ideal model system for the study of umbilical development for several reasons. First, formation of the placenta occurs on schedule in whole embryo culture of living mouse conceptuses (K.M. Downs and R.L. Gardner, supra, 1995; K.M. Downs, et al., supra, 1998). Second, the allantois, precursor of the umbilical cord, is particularly amenable to manipulation *in vitro* and can be isolated free of contamination from the conceptus (K.M. Downs and R.L. Gardner, supra, 1995; K.M. Downs and C. Harmann, supra, 1997; K.M. Downs, et al., supra, 1998; reviewed in K.M. Downs, supra, 1998). Third, transgenic mouse technology has enabled the identification of genes involved in formation of the placenta, either because its two major components, the allantois and the chorion, do not unite in the mutant mice (G.C. Gurtner, et al., Genes and Dev. 9:1-14, 1995; L. Kwee, et al., Development 121:489-503, 1995; J.T. Yang, et al., Development 121:549-560, 1995) or because vasculogenesis has not occurred in the umbilicus (R.J. Akhurst, et al., Development 108:645-656, 1990; M.C. Dickson, et al., Development 121:1845-1854, 1995; F. Shalaby, et al., Nature 376:62-66, 1995). Thus, the mouse is an ideal system in which to elucidate the genetic control of major developmental processes. There

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exists no other mammalian model at this time that exhibits all of these significant strengths.

At page 16, lines 15 - 28 and page 17, lines 1 - 11, please delete the paragraph and insert:

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In the course of our studies, we demonstrated that when allantoises are removed from headfold-stage conceptuses (approximately 8.0 days postcoitum) and cultured under relatively simple conditions in isolation, they rapidly undergo reproducible and stereotypic vasculogenesis (K.M. Downs, et al., supra, 1998). With feeding, the allantoic vasculature is maintained and remodeled for up to 3 days. The cultured explants consist of at least three cell lineages, endothelial, mesothelial, and mesenchymal, all of which are normally found in intact allantoises. Further, correct topographical relations between at least two of these lineages, the endothelial and mesothelial cells, are maintained in the explants. Moreover, cells from explanted cultured allantoises can be returned to developmentally-equivalent host allantoises where they correctly colonize appropriate cell types. Lastly, one of the explanted cell populations, the mesenchymal cells, can take up and express exogenous DNA. On the basis of our findings, we propose that the murine allantois will be a powerful and extremely valuable model system for at

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least two novel applications (Method 1 and Method 2),
described below:

At page 20, lines 3 - 28 and page 21, lines 1 - 6,
please delete the paragraph and insert:

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Vascular Endothelial Growth Factor (VEGF) is
expressed in the allantoic mesothelium (D.J. Dumont, et
al., Dev. Dyn. 203:80-92, 1995) before spreading into the
core (K.M. Downs, unpublished data). We have
demonstrated that culture of allantoic explants in high
rat serum (20-50% rat serum) is optimal for the formation
of blood vessels. Culture of explants in low serum
(fetal calf serum, FCS, 5-10%) favors formation of
angioblasts, as revealed by expression of Flk-1 and Flt-
1, early markers of angioblasts, but not their conversion
into nascent blood vessels. Moreover, despite feeding,
allantoises cultured in 5% FCS are typically devoid of
vascular channels by 48 hours. By 72 hours, explants
cultured in and fed 5% FCS at 24 hour intervals consist
predominantly of mesenchymal cells. Increasing the
concentration of FCS to 10-20% FCS results in partial
maintenance of vascular channels for up to 72 hours,
though significant breakdown of the channels is observed
in about 87.5% of explants. Thus, a high concentration
of some factor(s) must be required for both formation and
maintenance of endothelial cells in allantoic explants.

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To test that possibility, recombinant VEGF (1-10 ng/ml culture medium) was added to explants at the start of culture in 5% FCS. Feeding at 24 hour intervals in the presence of Vascular Endothelial Growth Factor (2-10 ng/ml) resulted in formation of many vascular channels containing Flk-1 and Flt-1, and cell survival (78% cell retention compared with 36% in untreated explants) whereas untreated explants or those treated with 1 ng/ml of VEGF were devoid of such channels.

At page 24, lines 26 - 28, page 25, lines 1 - 28, and page 26, lines 1 - 3, please delete the paragraph and insert:

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Calcium phosphate-mediated transfection involves mixing DNA directly with CaCl_2 and a phosphate buffer to form a precipitate that is added to the cultured cells. This method achieves both transient and stable expression of DNA, the latter following integration of the transfected DNA into the host cell genome (M. Wigler, et al., Cell 16:777-785, 1979; M. Botchan, et al., Cell 20:143-152, 1980; S. Kato, et al., Mol. Cell. Biol. 6:1787-1795, 1986) or by autonomous replication in mini-chromosomal structures (D.H. Hamer, et al., Cell 17:725-735, 1979; D. DiMaio, et al., Proc. Natl. Acad. Sci. USA 79:4030-4034, 1982; R. Reeves, et al., Nucl. Acids Res. 13:3599-3615, 1985). As described above, allantoises

will be removed and plated in individual wells of 24-well tissue culture dishes. One or more allantoises will be plated per well. Because CaP-mediated transfection requires that cells be 30-60% confluent, allantoises will be cultured for 12 hours, which is ample time for them to flatten out and spread somewhat on the bottom of the dish. Prior to transfection, the culture medium will be changed to medium containing 5% fetal calf serum, and the CaP/GFP complex added to the wells. The cells will be returned to the incubator and exposed to the precipitate for 6 hours, after which they will be washed with phosphate buffered saline (PBS, Sigma) and exposed to fresh media. They will then be re-fed every 24 hours up to the time of analysis, which will take place 36-54 hours after transfection (54-72 hours total time in culture). To increase the efficiency of transformation, some of the available "shock" methods, such as application of DMSO, will be applied to the cell cultures 14-16 hours after transfection and immediately removed and replaced with fresh medium.

At page 101, lines 1 - 28 please delete the paragraph and insert:

On the basis of gene expression in intact allantoises and morphology, we conclude that at least three major cell populations are present in explanted

allantoises: Flk-1-positive endothelial cells of the vasculature, VCAM-1-positive mesothelial cells overlying the vasculature, and a peripheral population of flattened mesenchymal cells that express neither flk-1 nor VCAM1. Downs, et al. have recently proposed that allantoic cells might be an attractive vehicle for the delivery of therapeutic factors to the fetal blood circulation in order to ameliorate certain developmental defects. Thus, to determine whether any of the three allantoic cell types could take up and express exogenous DNA, allantoises were transfected via the calcium phosphate precipitate method (F.L. Graham and A.J. van der Eb, supra, 1973) with a plasmid containing enhanced GFP expressed from the human cytomegalovirus early promoter.

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Cancel

In the Claims:

Please cancel claims 14, 17, 19, 20, 24, 25 and 26 and please add new claims 28 and 29. Please amend claim

27.

27. (Amended) The method of claim 25 wherein the test compound is a protein.

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28. (New Claim) A method of determining whether a compound affects vascularization of allantois tissue *in vitro* comprising the steps of:

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- a) isolating allantoic tissue,
- b) culturing said allantoic tissue *in vitro*,